SUPPLEMENT

Significance of 19-norandrosterone in athletes' urine samples

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Br J Sports Med 2006;**40**(Suppl I):i25–i29. doi: 10.1136/bjsm.2006.028027

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Nandrolone and other 19-norsteroid potent anabolic steroids have been prohibited in sports for 30 years. The detection of the main urinary metabolite—19-norandrosterone—in amounts greater than 2 ng/ml constitutes an adverse analytical finding. The presence in nutritional sport supplements of steroids not listed on the label has undoubtedly resulted in positive tests, but inadvertent consumption of meat containing residues of hormonal treatment should not realistically cause apprehension. Although highly improbable, athletes should prudently avoid meals composed of pig offal in the hours preceding the test since the consumption of edible parts of a non-castrated pig, containing 19-nortestosterone, has been shown to results in the excretion of 19-norandrosterone in the following hours. Norsteroid metabolites are formed during pregnancy and excreted as minor metabolites of norethisterone, and minute amounts have been identified in some male and female samples when using more sensitive techniques of detection. Whereas exercise does not seem to be a significant factor in 19-norandrosterone excretion, some rare urine samples were found to be a suitable medium for in situ 19-demethylation of urinary metabolites.

Nandrolone (19-nortestosterone, 17β-hydroxyestr-4-en-3-one) was first synthesised in the 1950s.[1] Its anabolic properties have prompted investigations to assess its clinical potential in male fertility control,[2 3] patients on haemodialysis,[4–7] aplastic anaemia (its main legitimate use),[8] asthenia in AIDS patients,[9] recovery following trauma or surgery,[10] protecting the immune system following cancer treatments,[11 12] osteoporosis,[13 14] and cachexia.[15] Anabolic steroids and 19-nortestosterone are used in medical veterinary practice for retarding degenerative processes and promoting tissue repair; they are also used—legitimately in some countries but not in others—as growth promoters in the farming industry. Nandrolone is perhaps best known as a performance enhancing substance, not devoid of adverse health effects, for increasing muscle strength and mass, and to speed up recovery, which it found its way in the sporting world (human and animal) as soon as it became available.[16–18] Surveys revealed use of anabolic steroids among adolescents not only for performance purposes but also to improve body image.[19 20] The use of anabolic androgenic steroids has been prohibited in ''amateur'' sports since the 1970s. A vast number of 19-nortestosterone human and veterinary preparations are available worldwide, most frequently for parenteral administration (intramuscular), as long-chain ester derivatives in the 17-O position (phenpropionate, decanoate) in vegetable oil; the pharmacokinetics are well described.[21] Further to the adoption of the Dietary Supplement and Health Education Act in the USA, several steroids and prohormones became available for oral self-administration worldwide and on the internet, including 19-norandrostenedione (estr-4 (or -5)-en-3,17-dione) and 19-norandrostenediol (3,17-dihydroxyestr-4 (or -5)-en); since January 2005 the distribution of those commercial products has finally been regulated/prohibited in many countries. Nandrolone and other 19-norsteroids have been banned to athletes for years and are listed in section S1, anabolic agents in the World Anti-Doping Agency list of prohibited substances and methods.[22]

NORSTEROIDS: METABOLISM, EXCRETION, AND DETECTION IN HUMAN URINE SAMPLES

The principal urinary metabolites formed following the administration of 19-nortestosterone (19-NT), were rapidly identified as 19-norandrosterone (19-NA; 3α-hydroxy-5α-androstan-17-one), 19-noretiocholanolone (19-NE; 3α-hydroxy-5β-androstan-17-one) and 19-norepiandrosterone (19-NEA; 3β-hydroxy-5α-androstan-17-one). The latter, possessing a 3β-hydroxyl group, is almost exclusively sulphoconjugated, whereas the other two are predominantly excreted as their glucuronide derivatives (fig 1).[23–25] The same metabolites are produced from ''prohormones'', 19-norandrostenedione and 19-norandrostenediol, often referred to as precursors of 19-NT.[26–28] When orally administered, due to extensive first pass metabolism, the metabolites are rapidly and massively excreted in the initial hours following administration. The enzymatic reactions involved are well known: phase I involving 5α- and 5β-reductases, 3α-, 3β-, and 17β-hydroxysteroid dehydrogenases, followed by phase II conjugative reactions with glucuronic acid or sulphate. Although after parenteral or oral administration of 19-NT and 19-norandrost-4-endione the formation of 19-NA (3α-OH, 5α-H) is clearly favoured over 19-NE (3α-OH, 5β-H), inverted ratios of the metabolites have been reported for 19-norandrost-4-en-3β,17β-diol, Δ^5-norsteroids and at the end of the excretion period following oral ingestion of nandrolone sulphate.[25–31]

The metabolites are easily detected in human urine samples following isolation through solid-phase extraction, enzymatic hydrolysis, liquid-liquid extraction, chemical formation of derivatives, usually per-trimethylsilylation, and gas chromatographic-mass spectrometric (GC-MS) analysis.[32–35] Parenteral administration of the long-chain esters of 19-nortestosterone may be detected for months, with anecdotal

Abbreviations: 19-NA, 19-norandrosterone; 19-NE, 19-noretiocholanolone; 19-NT, 19-nortestosterone; GC, gas chromatography; IRMS, isotope ratio mass spectrometry; LOD, limit of detection; MS, mass spectrometry; WADA, World Anti-Doping Agency

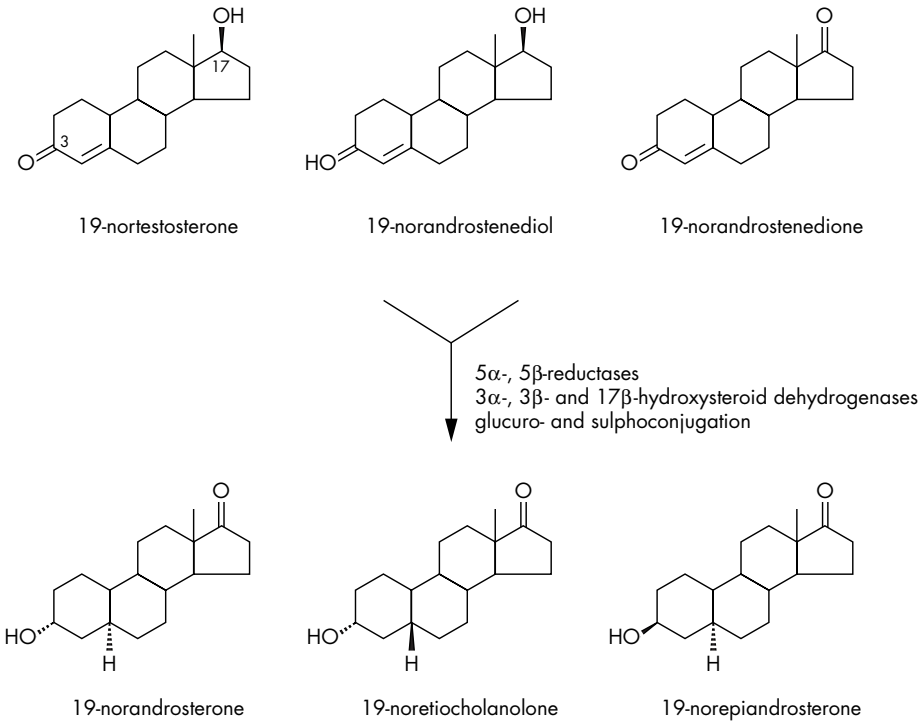

Figure 1 Metabolism of norsteroids in humans: 19-norandrosterone and 19-noretiocholanolone are predominantly glucuroconjugated, 19-epiandrosterone is sulphoconjugated.

evidence pointing to more than 18 months past the last injection. Metabolites formed after oral administration of norsteroids remain detectable for only a few days. Since strong interindividual variability exists in the excretion of metabolites linked to different rates of absorption and rapid elimination of metabolites following oral ingestion, it seems almost impossible to determine from the results of a single test what exact preparation was used, let alone the time, dosage,or mode of administration. It appears that high-intensity exercises do not influence the excretion of 19-norsteroids administered to trained athletes.[36]

The analysis of urinary metabolites by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been shown to be useful in determining their origin, endogenous or exogenous.[37–40] It is routinely used for confirmation in a few laboratories; major improvements were needed with regard to sample purification and instrumental conditions to reach the level of sensitivity required for the analysis of low levels of 19-norsteroid urinary metabolites.

19-NA IN ATHLETES' URINE SAMPLES

The Medical Commission of the International Olympic Committee (IOC) and, since 2003, the World Anti-Doping Agency (WADA) are collecting results of testing done on athletes' samples in accredited laboratories. Since 1988 it has appeared that testosterone, 19-nortestosterone (or precursors), stanozolol, and methandienone are the anabolic androgenic steroids most frequently found in athletes' samples. Each year, the presence of 19-NA is recurrently detected in an average of 0.23% of all samples tested (table 1).[41 42]

While testing athletes' samples, the laboratories must follow WADA's requirements as detailed in the International Standard for Laboratories including technical documents describing criteria for identification and reporting.[43] Adverse analytical findings occur when the concentration of 19-NA is greater than 2 ng/ml, with consideration being given to the estimated uncertainty of the measure; the threshold, introduced in 1998,[44] is adjusted to the specific gravity of the specimen. Verifications are made to exclude pregnancy and the legitimate use of norethisterone contraceptive medicines, which have been found to result in minor metabolites to low levels of 19-NA and 19-NE.

Table 1 Results of international testing in IOC/WADA accredited laboratories[41 42]

Year	Total number of tests	Nandrolone adverse findings reported	Per cent
1988	47069	304	0.65
1989	52371	224	0.43
1990	71341	192	0.27
1991	84088	165	0.20
1992	87808	152	0.17
1993	89166	227	0.25
1994	93680	207	0.22
1995	93938	212	0.23
1996	96454	232	0.24
1997	106561	262	0.25
1998	105250	259	0.25
1999	118259	293	0.25
2000	117314	325	0.28
2001	125701	304	0.24
2003	151210	256	0.17
2004	169187	339	0.20

19-NA NORMALLY FOUND IN HUMAN URINE SAMPLES

The threshold being fixed at 2 ng/ml, the methods used in laboratories should permit testing with a required performance level of 1 ng/ml.[45] It appears that the low excretion of endogenous 19-norandrosterone is usually not detected in human urine samples during routine doping control testing because the limit of detection (LOD) of the methods employed is usually in the order of 0.3–0.5 ng/ml. Exception should be made for specimens collected during pregnancy, in which levels can reach approximately 15 ng/ml.[46] The presence of 19-nortestosterone and 19-norandrostenedione in

human follicular fluids and of 19-nortestosterone in the plasma of pregnant women was reported in 1984 and 1987, respectively, but no 19-NT was detectable in the plasma of males and non-pregnant women. The hypothesis of a placental origin of 19-NT—for example, by an alternative aromatising pathway converting testosterone to estradiol by 19-demethylation, was presented.[47 48] The synthesis of 19-NT in the human ovary was described in 2002.[49]

More sensitive instrumentation such as GC or high performance liquid chromatography (HPLC) coupled with high resolution mass spectrometry or tandem mass spectrometry, larger volumes of urine, and extensive purification by HPLC or ion exchange and partition chromatography were needed to detect, identify, and quantify endogenous 19-norandrosterone. Between 1997 and 1999, three studies described under such experimental conditions, the presence of 19-NA in male specimens (sportsmen and volunteers). The levels were described at around 0.01–0.32 ng/ml (mean 0.08 ng/ml) in non-fractionated 24 hour urine,[50] 0.02 (LOD) to 0.6 ng/ml,[51] and lower than 0.5 ng/ml.[52] Although insulin stress had no effect on 19-NA excretion in males, gonadal stimulation with human chorionic gonadotropin was found to increase the level by 250%. The maximal concentration reached post-stimulation was 0.43 ng/ml.[53] In samples collected from non-pregnant females, the level of endogenous 19-NA seems also relatively low (below 1 ng/ml), varying during the menstrual cycle and correlating with higher plasma levels of 17β-estradiol,[54 55] or with increased excretion of luteinising hormone. In the latter study, a threefold to fourfold increase was measured with urinary concentrations peaking at a maximum of 0.8 ng/ml in mid-cycle.[56 57]

INGESTION FROM CONTAMINATED COMMERCIAL PRODUCTS

In the past five years, numerous studies have confirmed that products sold as nutritional or sport supplements are not properly labelled and contain, whether that is deliberate, steroids and prohormones including 19-norsteroids or stimulants such as caffeine and ephedrine, all of which do not appear on the list of ingredients. The ingestion of such products was shown to result in positive tests in the next hours.[58–65] Some athletes who tested positive were able to link the test result to a mislabelled commercial product.[66]

INGESTION OF NON-CASTRATED PIG OFFAL

Notwithstanding some reports suggesting a link existing between the consumption of meat contaminated by residual hormonal treatment and potential positive tests,[67 68] it is generally regarded as highly improbable since residues in meat, when measured, are very low; there is no indication that such a case happened.[50 51]

19-nortestosterone, the 17β-isomer, is present in the intact boar. A first report described the ingestion of a substantial amount of non-castrated pig meat (375 g) resulting in the excretion of 19-norandrosterone in amounts reaching 3–7.5 ppb in the following hours.[69] Another study confirmed that in spite of interindividual differences, 19-NA and 19-NE were always measured in specimens provided in the hours following the improbable ingestion (non-castrated pig meat being difficult to find) of such meat-rich meal composed of non-castrated pig tissues, with levels even reaching 160 ng/ml in one case. No trace of norsteroid metabolites was found in urine samples collected before or after the ingestion of castrated pig edible parts. As expected, the GC/C/IRMS analysis confirmed endogenous-like $^{13}C/^{12}C$ value of metabolites.[70]

THE EFFECT OF EXERCISE

In the past years, different groups have tested selected athletes' samples to determine whether exercise played a role in the excretion of norsteroid metabolites and apparently, it is not the case. One group analysed samples taken from two cohorts of football players, amateurs and professionals, and noted that up to 6% of the samples collected post-match—in uncontrolled conditions—contained traces of 19-NA; the authors prudently attributed the findings to either endogenous production, release from the fatty tissues of a previous intake of 19-NT, or intake just prior to the match of a product containing nandrolone.[71] Another group reported the results of 385 samples from 40 football players and described concentrations of 19-NA as significantly higher than those before match,[72] while again in uncontrolled conditions, traces of 19-NA were found in some professional football players' samples but were absent in samples from the control group.[73]

Under controlled conditions, baseline levels of 19-NA in male athletes have been found to be either undetectable or reaching a maximum level of 0.25 ng/ml (mean 0.048 ng/ml). Exercise was not found to influence 19-NT secretion or excretion of 19-NA.[74] There was no impact on the excretion of norsteroid metabolites of controlled exercise sessions in young male athletes; only one sample contained measurable amount of 19-NA (0.13 ng/ml).[75 76]

IN SITU 19-DEMETHYLATION OF URINARY STEROIDS

Recently, formation of trace amounts of norsteroid metabolites by 19-demethylation of etiocholanolone (3α-hydroxy-5β-androstan-17-one) and androsterone (3α-hydroxy-5α-androstan-17-one) was observed in athletes' samples following incubation. The reaction being favoured in 5β-isomers, 19-NA and 19-NE appear in ratios lower than that of their respective urinary precursors, androsterone and etiocholanolone.[77] Urine samples in which 19-demethylation activity was observed were often very concentrated, and turbidity was noted. This crucial observation, which was confirmed by two different groups, prompted the revision of the criteria for reporting adverse findings, to include verification steps in the relatively rare samples showing characteristic criteria of unstable urines.[78]

Efforts have been made to permit the reliable analysis of urinary 19-norandrosterone in trace amount by GC/C/IRMS which requires better instrumental sensitivity and improved

What is already known about this topic

Nandrolone and other 19-norsteroids have been prohibited in sports for 30 years. Although the detection of their main urinary metabolite, 19-norandrosterone, in amounts greater than 2 ng/ml constitutes an adverse analytical finding, there have been ongoing discussions on the influence of exercise as well as the consumption of nutritional supplements and pig offal on its excretion in the urine.

What this article adds

Nowadays, the use of IRMS identifies the endo- or exogenous origin of the urinary metabolites of 19-nortestosterone, even low quantities. Whereas exercise does not seem to significantly increase their excretion, athletes should refrain from taking nutritional supplements to avoid positive testing. Recently, the finding that in some unstable urine samples metabolites may be formed by in situ 19-demethylation, has prompted the inclusion of defined verification steps before reporting an adverse result.

sample purification. The origin of 19-NA present in amounts as low as 2–3 ng/ml can now be determined in a relatively low volume of urine, making it suitable for confirmation of athletes' samples (Hebestreit M *et al*, personal communication, 2006; Fakirian A *et al* unpublished data).

CONCLUSION

Administration of 19-nortestosterone does not represent a major analytical challenge and it has been tested relatively easily for almost 30 years in human athletes' urine through its characteristic metabolites, 19-norandrosterone and 19-noretiocholanolone. Norsteroids can be formed as side products during the conversion by aromatisation of steroids such as testosterone to estradiol and have been identified in some animal species, including humans, albeit in minute amounts. Following technical improvements in instrumental analytical technology rendering possible the detection of traces of metabolites, a threshold was fixed for 19-norandrosterone reporting. However, in recent years, the presence of 19-norsteroid metabolites in levels approaching and even exceeding the threshold were noted in some urine samples; the metabolites were formed by 19-demethylation of urinary steroids. IRMS provides conclusive evidence of the origin, endogenous or exogenous, of the urinary metabolites, even when found in low quantities.

Competing interests: none declared

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